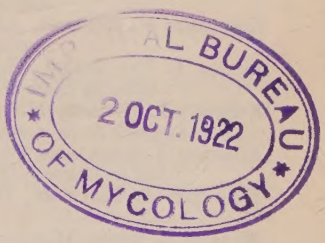
TECHNICAL BULLETIN No. 83 APRIL, 1921

New York Agricultural Experiment Station.

GENEVA, N. Y.

THE USE OF VARIOUS CULTURE MEDIA IN CHARACTERIZING ACTINOMYCETES

H. J. CONN



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* Connected with Grape Culture Investigations.
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TECHNICAL BULLETIN No. 83

THE USE OF VARIOUS CULTURE MEDIA IN CHARACTERIZING ACTINOMYCETES

H. J. CONN

SUMMARY

A study has been made of about 75 different types of soil Actinomycetes to determine the best media for use in studying them, and to learn the extent of variation in chromogenesis possible with the same culture under different conditions. Since this investigation was directed toward cultural characteristics alone, with no attempt to correlate them with morphology, no effort has been made to classify or to name the types studied.

For vigorous and characteristic growth of these organisms, a medium containing phosphate, glycerin, and sodium asparaginate is ordinarily sufficient. Media of other composition are often necessary, however, to bring out the most characteristic appearance of any species.

Extreme variation in chromogenesis is possible with some of the cultures studied, according to the composition of the medium, and some cultures even vary greatly when studied at different times on the same medium. The appearance of one of these organisms on any medium should not be described until it has been cultivated on several lots of this medium at different times. No culture, moreover, can be considered nonchromogenic until it has been studied on a great variety of different protein-free media.

INTRODUCTION

One of the most numerous and interesting groups of organisms found when studying soil by the culture-plate method is the group of Actinomycetes. As shown in previous papers,[1] their colonies may comprise as many as from 40 to 50 per cent of all the colonies developing on the plates. A large number of types of these organisms occur in the soil and, in respect to morphology and chromogenesis, they are unusually interesting, hence they have been given considerable study. In the soil flora studies,[2] previously undertaken in this laboratory,

[1] Conn, H. J. A possible function of Actinomycetes in soil. *N. Y. Agr. Exp. Sta. Tech. Bul. 52. 1916.*

Conn, H. J. Actinomycetes in Soil. *N. Y. Agr. Exp. Sta. Tech. Bul. 60. 1917.*

[2] Conn, H. J. Soil Flora Studies, I–V. *N. Y. Agr. Exp. Sta. Tech. Buls. 57, 58, 59, and 60. 1917.*

much attention was given to them and many facts accumulated which were not then published. At present, however, intensive work on the Actinomyces group must be dropped, and it seems worth while to publish such data as have been gathered, altho the work is incomplete and the conclusions indefinite. The largest part of the data at hand deals with the subject of the media best adapted to the study of these organisms.

HISTORICAL

Until recently there has not been much comparative data in regard to different media for Actinomycetes. They were at first grown largely on ordinary bacteriological agar media, presumably containing peptone and beef extract. There are frequent references to the use of glycerin agar,[3] but as its formula is not given, the assumption is that it was ordinary agar to which glycerin had been added.

Krainsky [4] seems to have been the first to call attention to the fact that the growth of these organisms on such media is non-characteristic, and that media containing no protein are much better adapted to bring out the striking characteristics of the different species. He used primarily a calcium malate agar and a dextrose agar. (See media 534 and 250, listed in Table 1 of this bulletin.) Waksman [5] and the writer [6] have followed up the idea of Krainsky and have added several new media to his list.

This recent work has brought out the fact that altho the saprophytic Actinomycetes can grow vigorously enough on or in media containing complex nitrogenous compounds like peptone, their growth on such media is seldom characteristic. The color attained on such media is generally yellow to buff with a white aerial mycelium, if any, while any color that diffuses in the media is ordinarily yellow or brown. These organisms, however, seem to thrive equally well with very small quantities of inorganic or amide nitrogen, provided some readily available source of carbon, such as sugar or glycerin, is present — glycerin proving especially favorable. On these simpler media the growth is very different from that on ordinary agar. Chromogenesis is frequent and is very striking. There are three different places where chromogenesis may be looked for: The mass of the growth before aerial hyphae are formed, the aerial mycelium,

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[3] See for example:

Savageau and Radias. Sur les genres Cladothrix, Streptothrix, Actinomyces. *Ann. Inst. Past.* 6, *242–273. 1892.*

Neukirch, H. Ueber Strahlenpilze. *Univ. Strassburg, Dissert. 1902.*

[4] Krainsky, A. Die Aktinomyceten und ihre Bedeutung in der Natur. *Centbl. Bakt. Abt II,* 41, *649–688. 1914.*

[5] Waksman, S. A. Cultural studies of species of Actinomycetes. *Soil Sci.* 8, *71–207. 1919.*

[6] See footnote 1.

and the medium itself as colored by soluble pigments diffusing thru it. Often the color is distinctly different in each of these three places, due sometimes, perhaps, to the presence of more than one pigment, but due more often to the different appearance of the same pigment when in solution, when in concentrated form in the mass of growth, or when in dry form within the spores. Differences in the composition of the media alter the chromogenesis, sometimes in a very striking manner.

There are no data in the literature to indicate whether the parasitic Actinomycetes grow as well on these simple media. A few cultures of animal parasites studied here did not do so. In other words, the saprophytic group are adapted to growth on simple compounds, while the parasites, if the indications are correct, have become so altered by their environment that they require organic nitrogen.

EXPERIMENTAL

PLAN OF EXPERIMENT

For this work about 75 cultures were selected from several soils to represent as many different types of Actinomycetes as were found in a brief study of the soils. The various media to be studied were inoculated two or three at a time, generally with all 75 cultures. They were not inoculated in duplicate because it was ordinarily very easy to detect a contamination, and nearly all of the media were inoculated more than once, thus making it possible to investigate disagreements. Before studying, the cultures were incubated three weeks at 25° C. The growth of each culture was then studied on each medium, a record made of chromogenesis and of other striking characteristics, and the medium which gave the most vigorous and that which gave the most characteristic growth with each culture noted. Any medium that gave promising results in either of these respects for any considerable number of the cultures was tested again in comparison with one or two other media.

In this way quite a large number of media were tested in the course of the work. At the beginning, one medium was chosen which was known to give good growth with a large number of the organisms, namely asparaginate glycerin agar of the formula already published.[7] This was first compared with simpler and simpler formulæ until an agar was obtained containing nothing but glycerin, water, and agar. Ingredients were then added, one by one, to this medium until the simplest medium was found which gave vigorous, characteristic growth with the majority of the organisms. Finally, still more ingredients were added in an attempt to find the most satisfactory formula. These tests are listed in Tables 2, 3, and 4, but not in the chronological order in which they were actually made.

[7] See N. Y. Agr. Exp. Sta. Tech. Bul. 60, p. 13.

MEDIA STUDIED

Such a large number of media were studied in the course of this work that it has seemed best to list their ingredients in the form of a table (Table 1), and also to adopt a decimal system of denoting them, giving each figure in the number of each medium a definite significance. Each medium has been given a number of three figures, the left hand figure designating the source of carbon, the second figure the source of nitrogen, and the right hand figure the non-nitrogenous mineral salts present. The system is as follows:

100 Glycerin
200 Dextrose
300 Glycerin and dextrose
400 Sucrose
500 Malic acid
600 Malic acid and glycerin
700 Citric acid and glycerin
800 Tartaric acid and glycerin
10 Sodium asparaginate
20 Ammonium phosphate (monobasic)
30 Ammonium chloride
40 Sodium asparaginate and ammonium phosphate
50 Sodium asparaginate and ammonium chloride
1 Potassium phosphate (dibasic)
2 Potassium chloride (with or without calcium chloride)
3 Potassium phosphate and calcium chloride
4 Potassium phosphate and calcium carbonate
5 Potassium phosphate and magnesium sulfate

Based on this system the asparaginate-glycerin-agar medium mentioned in the preceding paragraph is known as 342.

The media were divided into two series, designated as A and B. Series A included media 011 to 351, inclusive, which in general contained dextrose or glycerin as the main source of carbon, with no other organic matter except sodium asparaginate. One of the simpler media of this series, 001, did not contain either glycerin or dextrose, and some of the other simple media, including 100, 101, 120, and 201, did not contain sodium asparaginate. Series B included media 534 to 833, inclusive. These media contained as a source of carbon some hydroxy-acid, either malic, citric, or tartaric, alone or in addition to glycerin.

CULTURES USED

As already stated about 75 cultures were used. These were selected from about 300 cultures obtained from quite a number of different soils. While the majority of soils from which the cultures were secured were from the region of Geneva, N. Y., some were obtained

TABLE 1.— COMPOSITION OF THE VARIOUS MEDIA USED.

Besides ingredients listed, each medium contained 1000 cc. water.

	Medium number	Constituents of the media in grams per liter														
		Sources of carbon					Sources of nitrogen			Mineral ingredients						
		Glycerin	Dextrose	Malic acid	Citric acid	Tartaric acid	Sodium asparaginate	$NH_4H_2PO_4$	NH_4Cl	K_2HPO_4	KCl	$CaCl_2$	$CaCO_3$	$MgSO_4$	$FeCl_3$	Normal NaOH*
Series A	011						1.0			0.5						
	100	10														
	101	10								0.5						
	110	10					1.0									
	111	10					1.0			0.5						
	115	10					1.0			0.5						
	120	10						1.5								8 cc.
	140	10					1.0	0.5								3 cc.
	142	10					1.0	1.5			0.1					8 cc.
	151	10					1.0		0.5							
	155	10					1.0		0.5	0.5				0.2		
	201		10							0.5						
	211		10				1.0			0.5						
	242		10				1.5	1.5			0.2					8 cc.
	250		10				1.5		0.5							
	251		10				1.5		0.5	0.5						
	311	10	1				1.0			0.5						
	342	10	1				1.0	1.5			0.1	0.1			Trace	8 cc.
	351	10	1				1.0		0.5	0.5						
Series B	534			4					0.5	0.5			2.5			10 cc.
	554			4			1.0		0.5	0.5			2.5			10 cc.
	601	10		4						0.5						56 cc.
	610	10		4			1.0									56 cc.
	620	10		4				0.5								60 cc.
	631	10		4					0.5	0.5						56 cc.
	633	10		4					0.5	0.5		2.0				56 cc.
	634	10		4					0.5	0.5			2.5			10 cc.
	654	10		4			1.0		0.5	0.5			2.5			10 cc.
	731	10			4				0.5	0.5						53 cc.
	733	10			4				0.5	0.5		2.0				53 cc.
	751	10			4		1.0		0.5	0.5						53 cc.
	831	10				4.5			0.5	0.5						55 cc.
	833	10				4.5			0.5	0.5		2.0				55 cc.

* When the medium contained malic acid, citric acid, tartaric acid, or monobasic ammonium phosphate, enough normal NaOH was added to make the reaction essentially neutral, i. e. approximately pH=7.0.

from soils at quite different localities, namely Baltimore, Md.; Cambridge, Mass.; and Middletown, Conn.

These 300 cultures were first inoculated at the same time upon three different media, 342, 534, and 250. After growing from two to three weeks on these media, their growth was compared and they were grouped according to their appearance. No two cultures were put in the same group if they showed a different sort of growth on any one of the media. It was subsequently found that in some cases the subdivision had been carried too far on account of minor differences in appearance which were given more weight than they deserved; but, on the other hand, a considerable number of the groups would probably have been further subdivided if a larger number of media had been used. These groups varied in size from those containing but a single representative culture up to one group containing 36 cultures. One culture was selected from each of these groups for further study.

It cannot be claimed, of course, that in the 75 cultures thus selected every species present in the original 300 cultures, much less in the original soil samples, was represented, but it is felt that the 75 cultures so obtained were fairly representative of soil Actinomycetes in general. Media studies made with these selected cultures, while warranting no sweeping conclusions in regard to such a diverse group as the Actinomycetes, are nevertheless felt to have given very practical information as to the most satisfactory media to use in studying the types commonly encountered in the soil.

RESULTS

Tables 2 to 4 show the comparison of the different media obtained when tested with these cultures. In this work, sometimes two media and sometimes three were inoculated on the same day. When only two media were inoculated at once, one line in the table is given to each test. However, when three media were inoculated at the same time, as in Tests Nos. 2, 13, 14, etc., three comparisons instead of one were actually made, so that in such cases three lines are necessary in the table.

In each comparison there are enumerated first the number of cultures essentially alike on both media and then in the following columns, those cultures markedly different on the two media of each test, classified according to which medium gave the most favorable results. In thus analyzing the results, it was considered that there were two respects in which a medium might give more favorable results than the one with which it was being compared—it might allow more vigorous growth, or it might allow more characteristic growth than the other medium. Of these two bases of comparison, more characteristic growth was considered more significant than more

vigorous growth. Hence the cultures markedly different on the two media were classified into four main groups: (1) Those equally vigorous and characteristic on the two media, but differing in some other respect; (2) those more characteristic on one of the two media; (3) those more characteristic on the other medium; and (4) those equally characteristic on both media, but differing in vigor. These groups were further subdivided according to the relative vigor on the two media, as shown in Tables 2 to 4. This method of classification gives, in all, ten categories. In each comparison listed in the tables, the number of cultures falling in each of the ten categories are enumerated.

A discussion of the first few comparisons in Table 2 will make the matter clearer. In the first test, media 110 and 100 are compared, the former containing glycerin and sodium asparaginate and the latter only glycerin in addition to the agar and water. In this test, 71 cultures gave usable results, the others being excluded from the test for some reason such as contamination or failure to grow. Twenty-six of these cultures were essentially alike on both media, the other 45 being markedly different on the two media. Of the 45, 5 were equally vigorous and characteristic on both media. Twenty-seven were more characteristic on 110, 15 also being more vigorous than on 100, 11 equally vigorous on both, and 1 more vigorous on 100. Only two cultures were more characteristic on medium 100, neither of them being more vigorous than on 110, while one culture was less vigorous than on 110. Eleven were equally characteristic on both media, eight being more vigorous on 110 and three on 100. From this test, therefore, it was evident that the addition of asparaginate in general increased the vigor and the characteristic appearance of the cultures, altho over a third of the cultures grew alike on both media.

In the second test, three media, 110, 101, and 111, were studied this giving three comparisons 110 with 101, 110 with 111, and 101 with 111. All these media contained glycerin and medium 111 contained sodium asparaginate and potassium phosphate as well, while in 101 the asparaginate, and in 110 the phosphate was omitted. By far the best results in this test were obtained on medium 111, as will be seen in the table by noting the comparison of this medium with 110 and 101. There were 51 cultures more vigorous and characteristic on 111 than on 101, 44 more vigorous and characteristic than on 110; 10 more vigorous than on 101, but equally characteristic; and 13 more vigorous than on 110, but equally characteristic. On the other hand, while 11 were more characteristic on 101 and 16 on 110 than on 111, these were all less vigorous than on 111. There were no cultures alike on 110 and 111, or on 101 and 111; while only one culture was equally vigorous and characteristic on 110 and 101, differing in some other respect.

Turning now to the other comparison in this test, namely 101 with 110, it will be found that 11 cultures were essentially alike on both, while 5 were different, but equally vigorous and characteristic. Altho 24 cultures were more characteristic on 110 and 33 more characteristic on 101, in vigor both media gave essentially the same results. In brief, then, for the cultures studied all three ingredients, glycerin, phosphate, and asparaginate, were beneficial. The absence of either phosphate or asparaginate or both made the growth decidedly less vigorous or less characteristic. This method of comparison will serve as an illustration of the way to interpret the remainder of the tests in the three tables.

The tests listed in Table 2 were carried on with media of Series A, containing sodium asparaginate as their principal source of nitrogen and with glycerin or dextrose, or both, as sources of carbon. The first tests in Table 2 are interesting as they constitute a comparison between medium 111 and various other similar media in succession. Altho these other media were in some cases but slightly different from 111, there was not a single test in this series in which all of the cultures tested were alike on both media. There was not one of these similar media on which many of the cultures grew more vigorously than on 111, and, in general, no more cultures gave their more characteristic growth on these other media than on 111.

One medium investigated rather more than the others was 140, containing ammonium phosphate instead of potassium. It was finally concluded, however, that this medium gives no more valuable results than 111. In Test No. 14, medium 140 was compared with medium 342, the asparaginate glycerin agar of previous papers, and, as will be seen, rather more cultures gave their characteristic growth on the latter medium. Medium 342, therefore, is perhaps to be preferred to media 111 or 140, but the differences in its favor are not sufficient to justify the more complicated formula for routine use.

Tests Nos. 19 and 20 show the comparison of three media not included in any of the other tests, namely 250, 242, and 251. Of these three, 250 is the glucose agar used by Krainsky. In general it was found to give poorer results with the cultures tested than did the various glycerin media used. However, it happened to be valuable in just one case, namely in distinguishing a certain type of Actinomyces which was non-characteristic on other media, but which on this medium produced a striking violet halo beneath the surface of the agar. The two media with which medium 250 was compared in Tests Nos. 19 and 20 both contained phosphate, and were found, in general, to give better results, as can be seen from the table, but neither was of any use in distinguishing the particular type of Actinomyces which showed its characteristic chromogenesis on medium 250.

TABLE 2.— MEDIA OF SERIES A COMPARED ONE WITH ANOTHER AS TO THEIR SUITABILITY FOR THE CULTURES OF ACTINOMYCETES STUDIED.

TEST NO.	MEDIA COMPARED	TOTAL NUMBER OF CULTURES USED	NUMBER OF CULTURES ESSENTIALLY ALIKE ON BOTH MEDIA	NUMBER OF CULTURES MARKEDLY DIFFERENT ON THE TWO MEDIA								
				Equally vigorous and characteristic on both media*	More characteristic on first medium			More characteristic on second medium			Equally characteristic on both media	
					More vigorous on first medium	Equally vigorous on both media	More vigorous on second medium	More vigorous on second medium	Equally vigorous on both media	More vigorous on first medium	More vigorous on first medium	More vigorous on second medium
1	110 and 100	71	26	5	15	11	1	.....	1	1	8	3
2	110 and 101	73	11	5	1	23	.....	1	32	.....	.....	.....
	111 and 101	73	.....	1	51	.....	.....	.....	.....	11	10	.....
	111 and 110	73	.....	.....	44	.....	.....	.....	.....	16	13	.....
3	110 and 610	73	37	.....	2	12	.....	.....	10	.....	7	5
4	111 and 011	74	.....	.....	49	.....	.....	.....	.....	2	23	.....
5	111 and 311	71	51	.....	.....	6	.....	1	5	.....	5	3
6	101 and 201	71	36	1	2	12	.....	.....	10	1	9	.....
7	111 and 211	70	40	.....	.....	16	.....	.....	12	.....	.....	2
8	111 and 140	72	39	1	.....	16	1	.....	15	.....	.....	.....
9	111 and 151	73	34	3	.....	29	1	.....	6	.....	.....	.....
10	111 and 142	73	66	.....	.....	3	.....	1	3	.....	.....	.....
11	111 and 115	73	69	.....	.....	2	.....	.....	.....	.....	.....	2
12	140 and 120	73	3	1	26	10	.....	.....	3	5	25	.....
13	151 and 142	68	33	2	3	8	1	6	4	5	3	3
	151 and 140	71	19	1	10	5	.....	4	11	14	3	4
	140 and 142	70	18	2	2	11	4	10	5	7	7	4
	342 and 140	57	10	3	12	7	7	5	8	2	.....	1
14	342 and 155	56	6	.....	23	2	16	.....	1	8	.....	.....
	140 and 155	69	5	1	36	3	15	.....	2	7	.....	.....
15	342 and 151	75	63	1	.....	6	.....	.....	3	.....	1	1
16	342 and 151	9	4	1	.....	4	.....	.....	.....	.....	.....	.....
	342 and 351	9	4	1	.....	3	.....	.....	1	.....	.....	.....
	151 and 351	9	7	.....	.....	.....	.....	.....	2	.....	.....	.....
17	342 and 142	8	3	1	.....	3	.....	.....	1	.....	.....	.....
18	342 and 155	11	9	.....	.....	1	.....	.....	1	.....	.....	.....
19	250 and 242	56	4	.....	.....	10	9	10	4	.....	.....	19
20	250 and 251	71	6	2	.....	8	12	21	1	.....	.....	21

* The cultures enumerated in this column under each test, altho equally vigorous and characteristic on the two media, differed strikingly in chromogenesis, form of growth, or some other particular.

Table 3 gives the results with the hydroxy-acid media (Series B) together with a comparison of two malate media with 111 (Test No. 21). Those media containing malic acid were prepared to investigate Krainsky's claims as to the merits of this source of carbon for Actinomycetes. Medium 534 was an attempt to duplicate his calcium malate agar. Unfortunately at the time this work was done no calcium malate could be obtained commercially and it was prepared by neutralizing malic acid with calcium carbonate. This method of preparation gives the di-calcium salt, whereas Krainsky probably used the more soluble mono-calcium salt, as his formula calls for 10 grams per liter and the solubility of dicalcium malate is only about 0.8 per cent. Accordingly, the results obtained here are not strictly comparable with Krainsky's, as 0.5 per cent di-calcium malate instead of 1 per cent mono-calcium malate was used. In this work, malic acid was found to be a very good agent for bringing out characteristic features of chromogenesis, especially when combined with glycerin as in medium 634. Nevertheless, when this medium was compared with 111 (Test No. 21) rather better results were obtained on 111.

Tartaric and citric acids, also hydroxy-acids, were compared with malic acid in this work. Tartaric acid (Tests Nos. 22 and 23) proved practically, if not entirely unavailable, the growth on this medium being either scanty or absent with the majority of cultures. Citric acid, however, was not only a readily available source of carbon, but favored the production of fully as characteristic growth as did malic acid. This behavior of the two acids was a little unexpected, because at first thought tartaric acid seems more like malic acid chemically than does citric acid, as indicated by the following formulæ:

Malic acid, $COOH . HCOH . CH_2 . COOH$
Tartaric acid, $COOH . HCOH . HCOH . COOH$
Citric acid, $COOH . CH_2 . COH(COOH) . CH_2 . COOH$

Upon studying these formulæ more closely, however, it will be seen that citric acid, altho more complicated than either of the other two, is in one respect more like malic acid than is tartaric, namely in having the same series of radicals. That is malic and citric acids both contain the radicals COOH, COH, and CH_2, whereas tartaric acid contains only two of these, COOH and COH. It is possible that the combination of the hydroxy and methylene groups is necessary to make the compound physiologically available.

Citric acid cannot quite be substituted for malic acid in the study of the Actinomycetes, because certain organisms are more characteristic on one and some on the other, but for a routine medium it is fully as satisfactory and is more easily prepared. Altho citrate

TABLE 3.— MEDIA OF SERIES B COMPARED ONE WITH ANOTHER AS TO THEIR SUITABILITY FOR THE CULTURES OF ACTINOMYCETES STUDIED.

TEST NO.	MEDIA COMPARED	TOTAL NUMBER OF CULTURES USED	NUMBER OF CULTURES ESSENTIALLY ALIKE ON BOTH MEDIA	NUMBER OF CULTURES MARKEDLY DIFFERENT ON THE TWO MEDIA							
				Equally vigorous and characteristic on both media*	More characteristic on first medium			More characteristic on second medium			Equally characteristic on both media
					More vigorous on first medium	Equally vigorous on both media	More vigorous on second medium	More vigorous on second medium	Equally vigorous on both media	More vigorous on first medium	More vigorous on first medium	More vigorous on second medium
21	111 and 620	73	18	3	16	11	.....	1	8	6	10	.....
	111 and 634	72	8	.....	24	7	.....	4	15	9	2	3
	620 and 634	72	9	1	17	6	1	12	11	9	3	3
22	634 and 831	73	17	3	17	18	.....	.....	9	4	4	.....
23	634 and 833	71	6	2	32	21	.....	.....	4	1	4	1
24	634 and 633	67	41	2	.....	11	.....	.....	13	.....	.....	.....
	634 and 733	67	35	9	.....	5	.....	.....	18	.....	.....	.....
	633 and 733	69	41	4	.....	6	.....	.....	18	.....	.....	.....
25	634 and 631	73	43	2	.....	13	.....	.....	14	.....	1	.....
	634 and 731	73	34	2	.....	17	.....	.....	19	.....	1	.....
	631 and 731	74	48	2	.....	11	.....	.....	12	.....	1	.....
26	534 and 554	72	62	1	.....	8	.....	.....	1	.....	.....	.....
27	634 and 654	67	50	1	.....	7	.....	.....	8	.....	.....	1
28	731 and 751	71	41	2	.....	20	1	.....	6	.....	.....	2
29	631 and 601	71	4	2	38	5	.....	.....	.....	3	19	.....
30	620 and 601	64	3	.....	35	3	.....	.....	.....	2	21	.....
31	631 and 620	72	31	2	.....	11	1	7	12	.....	2	6

* The cultures enumerated in this column under each test, altho equally vigorous and characteristic on the two media, differed strikingly in chromogenesis, form of growth, or some other particular.

and malate media were not found to give better results with the majority of the cultures than did medium 111, nevertheless in some cases they brought out such striking features in the way of chromogenesis that one of them seems worth while recommending for general use. Perhaps the best malate formula is 634, and the best citrate formula 731.

SIGNIFICANCE OF MINUTE VARIATIONS IN THE FORMULA

In the course of this work some marked differences were observed when the formula of the medium was but slightly changed, and the question was raised as to how slight the variation need be to affect growth distinctly. Tests Nos. 32 to 34, listed in Table 4, were made with three different media having two different reactions, one about neutral and the other distinctly acid. It will be seen that with the dextrose media (250 and 242) some cultures grew more vigorously at one reaction and some at the other reaction; but that with the malate medium (654) more than half of the cultures grew more vigorously at the acid reaction. In the latter case, there was considerable doubt, however, as to whether the better growth at the more acid reaction was due to the reaction alone, or whether the increased acidity made the malic acid more available by converting it into some other chemical form.

An even more striking case of variation due to minute differences in the medium was observed in one test not recorded in the tables. In this case two lots of medium 731, alike in formula and reaction, were used, but one lot was made up with ordinary best quality shredded agar and the other with the granular, purified "Bacto-agar" of the Digestive Ferments Company. Altho there was no difference in the vigor of any culture on these two lots of media, 22 cultures were more characteristic with the shredded agar and only 8 more characteristic with the Bacto-agar, while 2 cultures were distinctly different on the two lots of media but were equally characteristic on both. Only 42 cultures, i. e. but a little over one-half of all the cultures examined, were essentially alike on the two lots of media. This difference between the two kinds of agar was presumably due to the influence of the impurities in the agar, of which more were undoubtedly present in the shredded agar than in the Bacto-agar.

The influence of minute variations is further illustrated in Tables 5 and 6. These tables show the appearance of the growth of two different cultures on the whole series of media used in these tests. Some of the media were tested several times, others only once or twice. It is interesting to compare the type of growth on the same medium at different times and on two media of similar composition. The two cultures selected for these tables were ones showing the most striking variations; so that while they are not to be regarded

TABLE 4.— COMPARISON BETWEEN TWO DIFFERENT REACTIONS IN CERTAIN MEDIA AS TO THEIR SUITABILITY FOR THE CULTURES OF ACTINOMYCETES STUDIED.

TEST NO.	MEDIUM USED	REACTIONS COMPARED EXPRESSED IN pH-VALUES	TOTAL NUMBER OF CULTURES USED	NUMBER OF CULTURES ESSENTIALLY ALIKE AT BOTH REACTIONS	NUMBER OF CULTURES MARKEDLY DIFFERENT AT THE TWO REACTIONS							
					More characteristic on more alkaline medium			More characteristic on more acid medium			Equally characteristic at both reactions	
					More vigorous on more alkaline medium	Equally vigorous at both reactions	More vigorous on more acid medium	More vigorous on more acid medium	Equally vigorous at both reactions	More vigorous on more alkaline medium	More vigorous on more alkaline medium	More vigorous on more acid medium
32	654	6.6 and 5.5.....	59	7	.... ..	2	4	6	1	.....	.....	39
33	250	7.4 and 5.5.....	69	32	1	.....	.....	1	.....	.....	12	23
34	242	7.0 and 6.0.....	69	39	1	3	.....	2	.....	3	18	3

as typical, they do show what may be expected with such organisms as these. In Table 5, it will be seen that the culture, one which has never been named, produced red growth on some media with a reddish or yellowish soluble pigment; while on other media the growth was gray to green or blue, or even black, with a greenish soluble pigment. On one or two media, (e. g. 111 and 731) both types of growth were observed.

The culture giving the results listed in Table 6, *Actinomyces violaceus-ruber* Waksman and Curtis, a strain furnished by Dr. Waksman, showed similar variations in chromogenesis; but in this case the two colors were red and blue, both colors transfusing thru the medium as well as thru the mass of growth. A careful inspection of these two tables will show many very striking differences due to slight changes in the formula of the medium.

Some of these differences are difficult to explain. One theory suggested is that the pigment produced may vary in color according to the reaction. This may be the explanation, but if so it is the final hydrogen-ion concentration and not the initial reaction that determines the color, for the color is not dependent upon the initial reaction in the case of media 242, 250, and 654, where two different reactions were tested. The final reaction is difficult to test in the case of such highly pigmented cultures, so that this point has not been investigated. No better explanation is offered; but a careful study of Tables 5 and 6 seems to indicate that each organism is capable of producing two distinct pigments, both separately or simultaneously, rather than one pigment alone which assumes two different color manifestations according to the cultural conditions.

Table 7 is added as a contrast to Tables 5 and 6. This table contains a similar description of the chromogenesis of *A. pheochromogenes* Conn,[8] an organism showing about the smallest amount of variation of any of the chromogenic cultures studied. Table 7 also offers a further contribution to the characterization of this species, previous descriptions of the organism consisting merely of a short paragraph in the bulletin cited and a slightly longer outline in a recent paper by Waksman.[9] A glance at Table 7 is sufficient to show that in every respect, except in regard to aerial mycelium, the chromogenesis is very constant. On some media, such as 011, 831, and 833, the chromogenesis is faint, but this is plainly due to poor growth, these media apparently containing no available source of carbon

[8] See N. Y. Agr. Exp. Sta. Tech. Bul. 60, p. 16.
[9] See footnote 5.

TABLE 5.— VARIATIONS IN CHROMOGENESIS AS SHOWN BY ONE OF THE CULTURES STUDIED AT VARIOUS DATES ON VARIOUS MEDIA.

MEDIUM	DATE	COLOR OF		
		Mass of growth	Aerial mycelium	Medium
342.....	April 12, 1915	Dark red.....	Gray-white.....	Dark brown
	June 26, 1915	Dark red.....	Gray-white.....	Red-brown
	Nov. 10, 1915	Dark red.....	Pink.....	Reddish
	June 21, 1916	Dull red.....	White*.....	Brownish
	Aug. 24, 1916	Dull red.....	Pink*.....	Yellow
	Aug. 31, 1916	Dark red.....	Gray to pink.....	Yellowish
	Sept. 6, 1916	Red.....	Gray-white.....	Yellow
342†.....	Nov. 10, 1915	Purple to gray...	Greenish gray....	Red-brown
151.....	June 21, 1916	Dark Green.....	White*.....	Dark Green
	Aug. 24, 1916	Dark Green.....	Gray.....	Dark Green
	Feb. 28, 1918	Very dark green..	White*.....	Grayish brown
	Oct. 31, 1918	Green to black...	———‡	Dark green
100.....	Aug. 9, 1917	Red.....	———	Colorless
110.....	Aug. 9, 1917	Dark green.....	Blue-green*.....	Green
	Aug. 23, 1917	Dark blue-green..	———	Green
	Sept. 5, 1917	Green.....	———	Yellow-green
101.....	Aug. 23, 1917	Dark red.....	———	Colorless
	Jan. 17, 1918	Pink and purple..	———	Purple
111.....	Aug. 23, 1917	Gray.....	———	Grayish brown
	Dec. 18, 1917	Red to purple....	———	Purplish gray
	Jan. 3, 1918	Dark brown.....	Gray-white to brown.....	Dark brown
	Jan. 31, 1918	Brown.....	Buff.....	Brown
	Feb. 14, 1918	Buff and dark red.	———	Dull red-brown
	Feb. 28, 1918	Black.....	Pink and white...	Grayish brown
	Mar. 28, 1918	Green to purplish.	Dull red.....	Brown
	April 25, 1918	Dark blue.....	Dull pink.....	Dark brown
	Nov. 29, 1918	Cream color, with red spots.....	———	Colorless
011.....	Dec. 18, 1917	Colorless.....	———	Colorless
311.....	Jan. 3, 1918	Dark brown.....	Dark brown.....	Dark brown
201.....	Jan. 17, 1918	Pink.....	———	Bluish

* Aerial mycelium scanty.

† This lot of medium 342 contained only 0.5 per cent instead of the usual 1 per cent of glycerin.

‡ A dash in this column signifies absence of aerial mycelium.

TABLE 5 (*continued*).

MEDIUM	DATE	COLOR OF		
		Mass of growth	Aerial mycelium	Medium
140.....	Feb. 14, 1918	Gray and light red	———‡	Green
	May 9, 1918	Buff and pink....	———	Green
	Oct. 31, 1918	Buff to light green	———	Light green
115.....	April 25, 1918	Dark blue.....	Dull pink.....	Dark brown
120.....	May 9, 1918	Buff and pink....	———	Colorless
142.....	Aug. 31, 1916	Almost black....	Gray-White.....	Dark brown
	Mar. 28, 1918	Green to purplish.	Dull red.....	Brown
	Oct. 31, 1918	Buff to green to black.....	———	Dark green
155.....	Sept. 6, 1916	Red.....	———	Yellowish or none
251.....	Mar. 9, 1917	Dark green.....	Gray.....	Green
250§.....	Mar. 9, 1917	Dark green.....	———	Green
	May 3, 1917	Blue.....	———	Green
	May 3, 1917	Dark green to blue	———	Green
242§.....	April 20, 1917	Almost black....	Gray-white.....	Green
	April 20, 1917	Dark gray-green..	Gray-white.....	Green
211.....	Jan. 31, 1918	Brown.....	Buff.....	Greenish
610.....	Sept. 5, 1917	Blue-green.....	———	Blue-green
634.....	Sept. 14, 1916	Dark gray.....	Greenish-gray....	Dark gray-green
	Sept. 28, 1916	Dark gray.....	Blue-gray.....	Dark gray-green
	Oct. 13, 1916	Dark green.....	Blue to gray.....	Dark gray-green
	Nov. 9, 1916	Dark green.....	———	Dark gray-green
	Nov. 29, 1918	Dark blue.....	———	Greenish brown
831.....	Sept. 14, 1916	Dull red.....	———	Greenish
833.....	Sept. 28, 1916	Red.....	———	Colorless
633.....	Oct. 13, 1916	Dark green.....	———	Brownish
733.....	Oct. 13, 1916	Red.....	———	Yellow
631.....	Nov. 9, 1916	Purple.....	Gray*.....	Blue and green
	May 23, 1918	Dark green.....	White.....	Dark green

* Aerial mycelium scanty.

‡A dash in this column signifies absence of aerial mycelium.

§ The lots of media 250 and 242 thus indicated were of an acid reaction, having a pH-value between 6.0 and 5.5.

TABLE 5 (*concluded*).

MEDIUM	DATE	COLOR OF		
		Mass of growth	Aerial mycelium	Medium
731.....	Nov. 9, 1916	Green and red....	White*.....	Green
	Nov. 16, 1916	Red.....	———‡	Not recorded
	Nov. 16, 1916	Dark green.....	———	Not recorded
	Mar. 22, 1917	Green and red....	———	Green
534.....	May 3, 1915	Dark green.....	Gray.....	Dark green
	June 26, 1915	Green.....	———	Not recorded
	Nov. 10, 1915	Green.....	Green and white..	Green
651§.....	April 5, 1917	Dark blue.....	———	Blue
	April 5, 1917	Dark green.....	———	Green and brown
751.....	Mar. 22, 1917	Buff.....	Gray-white.....	Brown
601.....	May 23, 1918	Blue.....	———	Blue
	Aug. 15, 1918	Dark blue.....	———	Dark blue
620.....	Aug. 15, 1918	Dark blue.....	———	Dark blue
	Aug. 29, 1918	Dark gray.....	———	Dark gray-green
	Nov. 29, 1918	Light blue.....	———	Blue

* Aerial mycelium scanty.

‡ A dash in this column signifies absence of aerial mycelium.

§ The lot of medium 651 thus indicated was of an acid reaction, having a pH-value between 6.0 and 5.5.

TABLE 6.—VARIATIONS IN CHROMOGENESIS AS SHOWN BY A SECOND CULTURE (*A. violaceus-ruber* WAKSMAN AND CURTIS) STUDIED AT VARIOUS DATES ON VARIOUS MEDIA.

MEDIUM	DATE	COLOR OF		
		Mass of growth	Aerial mycelium	Medium
342	Mar. 30, 1916	Red to buff	Buff	Orange
	June 21, 1916	Dull red	Gray	Orange
151	June 21, 1916	Dull red	Gray	Orange
	Feb. 28, 1918	Dark red	White*	Purple
	Oct. 31, 1918	Dark red	White*	Red
100	Aug. 9, 1917	Red	White	Colorless
110	Aug. 9, 1917	Blue	White	Blue
	Aug. 23, 1917	Blue	Blue*	Blue
	Sept. 5, 1917	Dark blue	Blue	Blue
101	Aug. 23, 1917	Pink	White*	Colorless
	Jan. 17, 1918	Red	Buff	Purple
111	Aug. 23, 1917	Dark blue	Pink	Purple
	Dec. 18, 1917	Purple	Pink	Purple
	Jan. 3, 1918	Purple	Gray to buff	Faint purple
	Jan. 31, 1918	Dark blue	Blue	Dark blue
	Feb. 14, 1918	Pink?†	Buff to pink	Pinkish
	Feb. 28, 1918	Dark red	White*	Purple
	Mar. 28, 1918	Red?†	Pink and gray	Brownish
	April 25, 1918	?†	Bright red and white	Pinkish
	Nov. 29, 1918	Red	White	Pinkish
011	Dec. 18, 1917	Colorless	White	Blue
311	Jan. 3, 1918	Dark brown	Gray to buff	Faint purple
201	Jan. 17, 1918	Violet	Gray	Blue
140	Feb. 14, 1918	Blue?†	Blue to gray	Dark blue
	May 9, 1918	Blue?†	Light blue	Blue to purple
	Oct. 31, 1918	Dark blue	Gray	Blue to purple
115	April 25, 1918	?†	Bright red and white	Pinkish
120	May 9, 1918	Pink	Buff	Colorless
142	Mar. 28, 1918	Red?†	Pink and gray	Brownish
	Oct. 31, 1918	Dark red	Gray*	Red

*Aerial mycelium scanty.

†Growth so covered with aerial mycelium at time of examination that color could not be determined.

TABLE 6 (*continued*).

MEDIUM	DATE	COLOR OF		
		Mass of growth	Aerial mycelium	Medium
155.....	Dec. 12, 1918	Pink.....	Pink to red.....	Colorless
251.....	Mar. 9, 1917	Dark blue.....	Gray.....	Blue
250§.....	April 19, 1916	Violet.....	White.....	Blue
	Feb. 1, 1917	Purple.....	?*.....	Pinkish
	Mar. 9, 1917	Dark blue.....	Blue?*.....	Blue
	May 3, 1917	Blue.....	Gray*.....	Blue
	May 3, 1917	Blue and purple..	Gray.....	Blue
242§.....	Feb. 1, 1917	Purple.....	Gray-white.....	Pinkish
	April 20, 1917	Dull red.....	———‡	Reddish
	April 20, 1917	Bright red.....	———	Reddish
211.....	Jan. 31, 1918	Dark red.....	Pink to gray.....	Pinkish
Czapek¶..	April 19, 1916	Blue and red.....	Gray.....	Yellowish
610.....	Sept. 5, 1917	Purple.....	Blue to gray.....	Blue
634.....	April 19, 1916	Red to violet....	White.....	Brownish
	Sept. 14, 1916	Dark purple.....	Gray-white.....	Purplish
	Sept. 28, 1916	Purple to red....	Gray-white*.....	Purplish
	Oct. 13, 1916	Purplish red.....	Pink.....	Reddish
	Nov. 9, 1916	Blue.....	Gray and blue....	Blue
	Feb. 15, 1917	Blue.....	Gray.....	Blue to brown
	Nov. 29, 1918	Blue.....	Blue-white.....	Blue
831.....	Sept. 14, 1916	Light red.....	———	Colorless
833.....	Sept. 28, 1916	Light red.....	———	Colorless
633.....	Oct. 13, 1916	Purple.....	Blue to gray.....	Dark blue
733.....	Oct. 13, 1916	Blue.....	Blue-gray.....	Dark blue
631.....	Nov. 9, 1916	Blue.....	Gray and blue...	Blue to red
	May 23, 1918	Blue?†.....	Gray.....	Dark blue
	Aug. 29, 1918	Blue?†.....	Gray to blue.....	Dark blue

* Aerial mycelium scanty.

† Growth so covered with aerial mycelium at time of examination that color could not be determined.

‡ A dash in this column signifies absence of aerial mycelium.

§ The lots of media 250 and 242 thus indicated were of an acid reaction, having a pH-value between 6.0 and 5.5.

¶ The medium was Czapek's solution agar as used by Waksman and Curtis, having the formula: Water, 1000 cc.: agar, 15 grams; $MgSO_4$, 0.5 gram; KCl, 0.5 gram; K_2HPO_4, 1 gram; $FeSO_4$, 0.01 gram; $NaNO_3$, 2 grams; and sucrose, 30 grams.

TABLE 6 (*concluded*).

MEDIUM	DATE	COLOR OF		
		Mass of growth	Aerial mycelium	Medium
731.....	Nov. 9, 1916	Blue.....	Gray and blue...	Blue to red
	Nov. 16, 1916	Blue.....	Gray and blue...	Blue to red
	Nov. 16, 1916	Red.....	Red.....	Not recorded
	Mar. 22, 1917	Blue to dull red..	Gray-white to red	Deep red
534.....	April 19, 1916	Red-brown†.....	Gray to green....	Colorless
	Feb. 8, 1917	?†.....	Gray to blue.....	Blue
554.....	Feb. 8, 1917	?†.....	Buff to blue.....	Blue
651§.....	Feb. 15, 1917	Blue.....	Gray and blue...	Blue to brown
	April 5, 1917	Dark blue.....	Blue and white...	Blue
	April 5, 1917	Dark blue.....	Blue.....	Blue
751.....	Mar. 22, 1917	Dull red.....	Red and blue*...	Deep red
601.....	May 23, 1918	Reddish purple...	Gray.....	Purple
	Aug. 15, 1918	Dark blue.....	———‡	Dark blue
620.....	Aug. 15, 1918	Dark blue?†.....	Purple.....	Dark violet
	Aug. 29, 1918	Purple?†.....	Gray to reddish..	Reddish
	Nov. 29, 1918	Blue.....	Blue and white...	Deep blue

* Aerial mycelium scanty.

†Growth so covered with aerial mycelium at time of examination that color could not be determined.

‡ A dash in this column signifies absence of aerial mycelium.

§ The lot of medium 651 thus indicated was of an acid reaction, having a pH-value between 6.0 and 5.5.

TABLE 7.— CHROMOGENESIS OF *A. pheochromogenus* ON VARIOUS MEDIA INOCULATED AT VARIOUS DATES.

MEDIUM	DATE	COLOR OF		
		Mass of growth	Aerial mycelium	Medium
342	—— —, 1916	Buff to brown	———*	Red-brown
	June 21, 1916	Buff to brown	———	Deep red-brown
	Dec. 12, 1918	Dark brown	———	Brown
151	June 21, 1916	Dark brown	White	Deed red-brown
	Oct. 31, 1918	Dark brown	Buff†	Brown
100	Aug. 9, 1917	Dark brown	———	Deep brown
110	Aug. 9, 1917	Dark brown	———	Deep brown
	Aug. 23, 1917	Dark brown	———	Brown
	Sept. 5, 1917	Dark brown	———	Deep red-brown
101	Aug. 23, 1917	Dark brown	Buff	Brown and green
	Jan. 17, 1918	Dark brown	White	Brown to green
111	Aug. 23, 1917	Dark brown	Buff	Brown
	Dec. 18, 1917	Dark brown	———	Deep brown
	Jan. 3, 1918	Dark brown	Buff	Deep brown
	Jan. 31, 1918	Dark brown	———	Dark brown
	Feb. 14, 1918	Dark brown	White	Deep brown
	Mar. 28, 1918	Dark brown	Buff to white	Dark brown
	May 16, 1918	Dark brown	Buff	Dark brown
	Nov. 29, 1918	Dark brown	White	Dark brown to yellow
011	Dec. 18, 1917	Colorless	White	Yellowish
311	Jan. 3, 1918	Dark brown	Buff	Deep brown
201	Jan. 17, 1918	Dark brown	White	Brown to green
140	Feb. 14, 1918	Dark brown	Buff	Light brown
	May 9, 1918	Buff	Buff to white	Brown
	Oct. 31, 1918	Dark brown	Gray-white	Brown
	Dec. 12, 1918	Brown?‡	Buff	Brown
115	April 25, 1918	Dark brown	Buff	Dark brown
120	May 9, 1918	Cream to buff	———	Brownish
142	Mar. 28, 1918	Dark brown	Buff to white	Dark brown
	Oct. 31, 1918	Dark brown	Buff and white	Brown

* A dash in this column signifies absence of aerial mycelium.

† Aerial mycelium scanty.

‡ Growth so covered with aerial mycelium at time of examination that color could not be determined.

TABLE 7 (*continued*).

MEDIUM	DATE	COLOR OF		
		Mass of growth	Aerial mycelium	Medium
155.....	Dec. 12, 1918	Brown to buff....	———*	Brown
251.....	Mar. 9, 1917	Dark brown.....	Pink-white.....	Red-brown
250‡.....	—— —, 1916	Red-brown.....	Buff.....	Brown
	Feb. 1, 1917	Dark brown.....	White†.....	Red-brown
	Mar. 9, 1917	Dark brown.....	White†.....	Red-brown
	May 3, 1917	Dark brown.....	White†.....	Red-brown
	May 3, 1917	Dark brown.....	White†.....	Red-brown
242‡.....	Feb. 1, 1917	Dark brown.....	Gray-white.....	Red-brown
	April 20, 1917	Dark brown.....	Buff.....	Deep red-brown
	April 20, 1917	Dark brown.....	White†.....	Deep red-brown
211.....	Jan. 31, 1918	Dark brown.....	White.....	Dark brown
610.....	Sept. 5, 1917	Dark brown.....	———	Deep red-brown
634.....	—— —, 1916	Dark brown.....	Buff and white...	Brown
	Sept. 14, 1916	Brown.....	Gray.....	Brown
	Sept. 28, 1916	Brown.....	Gray.....	Gray-brown
	Nov. 9, 1916	Dark brown.....	Gray.....	Dark brown
	Oct. 13, 1916	Dark brown.....	Gray.....	Dark brown
	Feb. 15, 1917	Dark brown.....	White.....	Deep red-brown
	Nov. 29, 1918	Dark brown.....	Buff†.....	Dark brown to yellow
831.....	Sept. 14, 1916	Buff (light).....	———	Colorless
833.....	Sept. 28, 1916	Cream.....	———	Colorless
633.....	Oct. 13, 1916	Dark brown.....	———	Dark brown
733.....	Oct. 13, 1916	Brown to buff....	———	Dark brown
631.....	Nov. 9, 1916	Dark brown.....	———	Dark brown
	May 23, 1917	Dark brown.....	———	Deep red-brown
731.....	Nov. 9, 1916	Dark brown.....	———	Dark brown
	Nov. 16, 1916	Dark brown.....	———	Dark brown
	Nov. 16, 1916	Dark brown.....	———	Dark brown

*A dash in this column signifies absence of aerial mycelium.

†Aerial mycelium scanty.

‡The lots of media 250 and 252 thus indicated were of an acid reaction having a pH-value between 6.0 and 5.5.

TABLE 7 (*concluded*).

MEDIUM	DATE	COLOR OF		
		Mass of growth	Aerial mycelium	Medium
534.....	—— —, 1916	Buff.....	Pink-white.....	Colorless
	Feb. 8, 1917	Brown? †.....	Pink-white.....	Brownish
554.....	Feb. 8, 1917	Brown? †.....	Pink-white.....	Brownish
651‡.....	Feb. 15, 1917	Dark brown.....	White.....	Deep red-brown
	April 15, 1917	Dark brown.....	Gray-white.....	Dark brown
	April 15, 1917	Dark brown.....	———*	Dark brown
601.....	May 23, 1917	Dark brown.....	———	Deep red-brown
620.....	Aug. 29, 1918	Dark brown.....	———	Dark brown
	Nov. 29, 1918	Dark brown.....	White.....	Dark brown to yellow

* A dash in this column signifies absence of aerial mycelium.

† Growth so covered with aerial mycelium at time of examination that color could not be determined.

‡ The lot of medium 651 thus indicated was of an acid reaction having a pH-value between 6.0 and 5.5.

CONCLUSIONS

The general conclusion to be drawn from this work is that much caution is necessary in making media for this group of organisms when the appearance of their growth on the media is to be used for the purpose of characterization. Pure chemicals and washed or specially purified agar should be used, and the quantities employed must be carefully measured. The reaction must be very carefully adjusted by the use of some indicator whose shade at the desired hydrogen-ion concentration is known. Whenever an organism is to be characterized in any publication by its growth on some medium, care must also be observed in describing the medium. The exact formula, reaction, and method of preparation must always be given. It is also necessary to study an organism on several different lots of any medium to determine the extent of variation before the appearance of the growth is used in characterizing the organism.

The formula which proved most satisfactory in this work as a medium for routine use in the study of this group of organisms was 111: Agar, 15 grams; glycerin, 10 cc.; dibasic potassium phosphate, 1 gram; sodium asparaginate, 1 gram; and water, 1000 cc. This was the simplest formula giving fairly characteristic and vigorous growth with the majority of the cultures tested. Altho not always giving the most characteristic growth obtainable, it is to be recommended chiefly on account of its simplicity, as it is advisable to use the fewest possible ingredients in the case of organisms so easily influenced by minute variations in the medium.

Other media should also be used to separate cultures not distinguishable by their growth on medium 111. For this purpose the malate (634) and citrate (731) media with glycerin are to be recommended. Another good medium for this purpose is the adaptation of Czapek's formula used by Waksman. (See footnote to Table 6.) Altho this was not tested in the comparative work here reported, it was omitted solely because of its more complicated formula, the object of the present work being to determine the simplest medium which would give general satisfaction. However, it was used and in several cases brought out some characteristic type of chromogenesis not observed on other media.

In searching for chromogenesis in this group, it cannot be said without investigation that any medium is of no value, nor can it be said that any culture is non-chromogenic until an extremely large number of different media have been used.